STUDIES OF MOLECULAR GENETIC DIVERSITY IN RICE WITH REFERENCE TO SALINITY TOLERANCE

By

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Synopsis

Abiotic stresses such as salinity, drought, low temperature, heavy metals etc. affect plant growth and development, resultantly affecting the crop productivity severely. Riceis one of the major agronomically and nutritionally important cereal food crops but is susceptible to the salinity. The recent advancement of plant biotechnology has opened two very important areas for crop improvements. One is Marker Assisted Selection (MAS) and second one is transgenic technology for introgressing the candidate genes in the susceptible background. Being a polygenic trait, salt tolerance is regulated by multiple factors. It is decisive to map the salinity-responsive QTLs with their linked markers, and then introgress them into the elite susceptible rice backgrounds to develop the salt tolerant rice varieties for boosting rice productivity in saline prone areas. Moreover, genes that contributeto the regulation of polygenic salt tolerance trait in plants are microRNA (miRNA) genes (Ren et al. 2013; Zhu et al. 2013). miRNAs are wide-spread class of newly uncovered RNAs, produced from non-protein-coding mRNAs which undergo various processing steps to form 21 to 26 bp long mature miRNAs. They silence the gene expression, at post-transcriptional level, in a sequence specific manner (Mondal and Ganie, 2014).

During my doctoral work, I mainly focussed on i) studying the molecular genetic diversity of *Saltol* QTL as well as different salt-responsive miRNAs in different rice genotypes differing in salt tolerance using the *Saltol* QTL-linked SSRs and miR-SSRs respectively ii) analysing the functional divergence of a salt-responsive (miRNA-target) module in 2 contrasting rice genotypes under salinity stress and iii) identifying and evolutionarily analysing the miRNA genes of different *Oryza* species.

I started with the assessment of the genetic diversity at *Saltol* QTL in a broad collection of rice genotypes including salt tolerant and susceptible rice landraces and varieties. Firstly, I found that a considerable variation occurs at this locus among the rice genotypes with wild rice and wild rice relatives having greater variation at this locus, indicating that the wild rice has a very high genetic potential at this locus also. These allelic variations in wild rice and other rice genotypes may prove as promising genomic resources for the improvement of rice salt tolerance in breeding programs. Secondly, I endeavoured to analyse the haplotypes of *Saltol* QTL on the basis of its four tightly linked SSR marker loci (RM1287, RM8094, RM562 and RM3412) in a rice germplasm comprising 142 diverse rice genotypes. A significant diversity of this locus in the germplasm was observed and led me to identify 10 different haplotypes with salt tolerant cultivar FL478 as a reference because this genotype has

already been found to possess the *Saltol* (Thomson et al. 2010). I found 68 rice genotypes possessing at least one allele of FL478 haplotype, and may be used as possible donors of this QTL for introgression purposes.

Next I turned my focus to studying the structural and functional diversity of salt-responsive miRNAs of rice. Although a larger number of SSRs are available, most of them are either from protein-coding regions or untranslated regions of rice genome. So, I thought that the development of novel markers from the conserved regions (regions containing miRNA genes) of rice genome will be suitable for studying the genetic diversity of closely related species or self- pollinated species. Therefore, I undertook a genome-wide SSR mining studywhich yielded me a total of 129 SSR markers from the entire miRNA genes of rice recorded in the miRBase, and validated 20 of them with repeat numbers ≥7 from all the 12 chromosomes among 24 diverse rice genotypes. I found that miRNA-based microsatellite marker system is very well proficient, novel and breeder friendly source for genetic diversity analysis or genotyping of rice. The markers developed are available at NCBI Probe (http://www.ncbi.nlm.nih.gov/probe) with the accession numbers (Pr032290639 to Pr032298077).

Since my focus was on salinity, I selected the SSRs, developed in the mentioned genome-wide study, from the salt-responsive miRNA genes of rice and studied their diversity in two contrasting panels of rice genotypes differing in response to salinity in order to gain the preliminary insight into the possible sequence variations in these genes. I found that the miRNA genes were more diverse in susceptible rice genotypes than the tolerant ones. This led me to hypothesize that higher repeat variations in the miRNA genes of salt-sensitive genotypes than tolerant ones might interfere with the formation of the stem-loop structure of precursor miRNA in susceptible genotypes, and hence with the subsequent synthesis and expression of the functional mature sequence. This was reasonable from the finding that SSR sequences can adopt a wide variety of unusual DNA structures with simple and complex hair pin or loop-folding patterns which may have important regulatory consequences for the gene expression (Fabregat et al. 2001). This however needs to be experimentally validated through further genomics studies. I found that simple sequence repeat (SSR) marker developed from miR172b gene sequence was useful in differentiating the tolerant and susceptible genotypes.

In continuation with the last study, I was enthusiastic to know how miRNAs may actually regulate the salinity tolerance trait differentially in salt-tolerant and salt-susceptible rice genotypes. I began this study by analysing the diversity in the promoter regions of some salt-responsive miRNA genes between the salt tolerant and sensitive rice genotypes. I found that

there was no considerable difference in the genetic variability in the promoter regions of salt-responsive miRNA genes between the 2 contrasting panels of rice genotypes, and that miRNA gene promoters were less variable than their corresponding coding regions (as mentioned above). Then I asked the question whether there was any difference in the mature miRNA sequence of a miRNA gene and/or its cleavage/target site between the salt tolerant and sensitive rice genotypes. For this, I selected (osa-miR393-TIR1) module. I cloned a small region (containing the mature sequence) of a salt-responsive miRNA gene (osa-miR393a) and its cleavage site in the target *TIR1* from the 5 salt tolerant and 5 salt susceptible rice genotypes. Interestingly, I found that both mature sequence as well as the cleavage site of osa-miR393a was completely conserved between the two panels of contrasting rice genotypes. Next, I endeavoured to know the any differences in the abundance of this miRNA as well as its target gene *TIR1* in salt tolerant FL478 and salt susceptible IR29 genotypes. I found that salt stress altered the expression pattern of osa-miR393a-TIR1 module in a time dependent manner in the roots and shoots of the two rice genotypes with the overall down regulation of miR393a and up-regulation of *TIR1* in FL478 and their reciprocal regulation in IR29. This was reasonable in the sense that osa-miR393a negatively regulates the salt tolerance in rice (Gao et al. 2011). I was curious to know if the difference in expression pattern of osa-miR393a in two contrasting rice genotypes under salt stress was due to some upstream processes like promoter methylation. Therefore, I undertook comparative methylation profiling in the promoters of osa-miR393a and *TIR1* in two contrasting rice genotypes FL478 and IR29 using EZ DNA methylation^{TM} Kit. Significant changes in promoter methylation were observed in a time dependent manner with the higher methylation in the osa-miR393a-promoter of FL478 than that of IR29, as vice-versa for *TIR1*. Hence, the expression results were complemented by the differential promoter methylation in the two rice genotypes. I also found differential *cis*-element abundance in the promoter regions of this regulatory module in two genotypes. Hence, differential promoter methylation and *cis*-element abundance might lie behind the differential expression of osa-miR393-TIR1 module under salinity in rice. Together, the results of transcript abundance and promoter methylation of miR393a-TIR1 module signified the association between these two processes under salinity, which was reported for the first time in plants.

At last I wanted to identify the miRNA genes from the different *Oryza* species for which I had to first identify the miRNA genes from these genomes. Therefore, orthologous miRNA genes of *O. sativa* (*japonica*) from these genomes were identified through bioinformatics approaches and the changes that might have occurred during their course of evolution were

analysed. Large size expansions, possibly owing to the tandem duplications, were observed in AA-genomes as compared to BB (*O. punctata*) and FF (*O. brachyantha*) genomes for the many miRNA gene families. The investigation of evolutionary rates indicated that conserved miRNA genes showed lesser substitution rates than the non-conserved miRNA genes across the *Oryza* species. Mature region had lesser substitution rates than the star and precursor sequences which inturn showed considerably lower rates than the synonymous and non-synonymous substitution rates in protein-coding genes. Evolution of *Oryza* genomes also seemed to be contributed by transposons. The neutrality tests suggested a non-neutral selection at the 86 selected miRNA loci across *Oryza* and these loci were estimated to have lost ~87% of the sequence diversity during the domestication. The phylogenetic analysis revealed that *O. longistaminata* was the earliest divergent species among the AA-genomes, whereas *O. brachyantha* and *O. punctata* appeared as the eminent out-groups. The miR1861 family arranged into nine distinct compact clusters in the studied *Oryza* species but was found absent in *O. brachyantha*. Its structure was conserved but was organised differently in *Oryza*. The gene expression analysis showed that the expression of 11 salt-responsive miRNAs was differentially regulated in tetraploid *O. coarctata* and diploid *O. glaberrima.* The evolutionary dynamics in the miRNA genes of different species of *Oryza* analysed in this study will support the more investigations about the improvement of rice breeding programs.

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